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Identification of suitable reference genes by quantitative real-time PCR for gene expression normalization in sunflower

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Supplementary Table 1. A checklist outlining the RNA to qRT-PCR quality/methodology as described in Bustin et al. (2009).

Item to check	Details
EXPERIMENTAL DESIGN	
Definition of experimental and control groups	Provided in the methods section.
Number within each group	Two different sunflower plants were used to obtained each independent biological replicate.
Assay carried out by core lab or investigator's lab?	Investigator's lab
SAMPLE	
Description	Provided in the methods section.
Volume/mass of sample processed	100-150 mg of leaves, roots, embryos and flowers samples were weigh in 1.5 ml tubes and frozen immediately with liquid nitrogen. Samples were processed in the same tube into frozen racks using steril micropestles.
Microdissection or macrodissection	
Processing procedure	

If frozen - how and how quickly?	
If fixed - with what, how quickly?	
Sample storage conditions and duration (especially for FFPE samples)	
NUCLEIC ACID EXTRACTION	
Procedure and/or instrumentation	According to PureLink™ RNA Mini Kit (Ambion by Life Technologies) manual (Cat no#12183018A). No modification and additional reagents were used.
Name of kit and details of any modifications	
Source of additional reagents used	
Details of DNase or RNAse treatment	DNAseI treatment was performed using the PureLink™ DNAse for use with in PureLink™ RNA Kit on column protocol (Invitrogen Life Technologies).
Contamination assessment (DNA or RNA)	No amplification was detected in no RT samples.
Nucleic acid quantification	Spectrophotometer
Instrument and method	A 5:50 dilution was measured with spectrophotometer PerkingElmer® Lambda Bio+.
Purity (A260/A280)	The A260/280 ratio was generally 1.7 to 2.5 and the A260/230 was between 1.5 and 2.0.
Yield	About 80 to 180 ng/µl from each RNA extraction (volume 100 µl).
RNA integrity method/instrument	The RNA integrity was confirmed by 1% agarose gel electrophoresis gel stained with SYBR® Safe- DNA Gel Stain (Thermo Fisher Scientific).
RIN/RQI or Cq of 3' and 5' transcripts	
Electrophoresis traces	
Inhibition testing (Cq dilutions, spike or other)	No inhibition was confirmed by inhibition testing with Cq dilutions.
REVERSE TRANSCRIPTION	
Complete reaction conditions	According to SuperScriptII® First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies).
Amount of RNA and reaction volume	20 µl reaction volume containing 0.5 µg of total RNA.
Priming oligonucleotide (if using GSP) and	5 µM of oligo dT primer

concentration	
Reverse transcriptase and concentration	10 U/µl of Reverse Transcriptase from SuperScriptII® First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies).
Temperature and time	42°C for 1 hour
Manufacturer of reagents and catalogue numbers	SuperScriptII® First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) (Cat no#18064014).
Cqs with and without RT	The amplicon of ACT gene was used for the evaluation of the cDNA quality of each retrotranscription and in this case the Cqs with RT were about 20-24 (with an average of Amplification Factors of 1.82), without RT no amplification was detected.
Storage conditions of cDNA	Stored at -20°C
RT-qPCR TARGET INFORMATION	
If multiplex, efficiency and LOD of each assay.	Multiplex qPCR was not performed.
Sequence accession number	Shown in Table 1
Location of amplicon	Exons
Amplicon length	Shown in Table 1
In silico specificity screen (BLAST, etc) Pseudogenes, retropseudogenes or other homologs? Sequence alignment	Amplicons were not sequenced.
Secondary structure analysis of amplicon	Not analyzed
Location of each primer by exon or intron (if applicable)	Exons
What splice variants are targeted?	No variant
RT-qPCR OLIGONUCLEOTIDES	
Primer sequences	Shown in Table 1

Specificity analysis of oligonucleotides	Primer specificity was checked against *Helianthus annuus* (taxid 4232) nr database (non redundant sequences) form NCBI's Nucleotide collections (www.ncbi.nlm.nih.gov)
RTPrimerDB Identification Number	None
Probe sequences	None
Location and identity of any modifications	None
Manufacturer of oligonucleotides	Oligo Macrogen - Macrogen Inc. (Korea)
Purification method	Salt-free
RT-qPCR PROTOCOL	
Complete reaction conditions	Described in the methods section.
Reaction volume and amount of cDNA/DNA	15 µl reaction volume containing 2 µl of cDNA reaction mixture.
Primer, (probe), Mg^{++} and dNTP concentrations	dNTP, Mg^{++}, buffer, and Taq DNA Polymerase were included in the Mezcla Real qPCR®, Biodynamics, Argentina (Cat no# B124-100), concentrations were not available.
Polymerase identity and concentration	
Buffer/kit identity and manufacturer	
Exact chemical constitution of the buffer	
Additives (SYBR Green I, DMSO, etc.)	SYBR Green™ was included in the Mezcla Real qPCR®, Biodynamics, Argentina
Manufacturer of plates/tubes and catalog number	qPCR strip tubes + caps 0.1 mL x 4 for Corbett RG-6000 and Qiagen RG-Q, Axygen™ (Cat no# PCR-0104-C).
Complete thermocycling parameters	95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min, followed by dissociation step [95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec]
Reaction setup (manual/robotic)	Manual
Manufacturer of qPCR instrument	Qiagen (Rotor-Gene® Q with HRM- Hight Resolution Melting)
RT-qPCR VALIDATION	

Specificity (gel, sequence, melt, or digest)	Specific amplicons were confirmed by the presence of a single peak in qRT-PCR melting curve. Melting rate was developed from 72 to 95°C with a ramp rate of 0.2°C/cycle, holding 5 secs on each step. Melting curves were visualized with the Melting application of the Rotor-Gene Q Series Software 1.7 (Build 94) Copyright ® 2008 Corbett Life Science. All melting curves are shown in the panel A of ESM2. The products were confirmed by size estimations on a 2.5% agarose gel stained with SYBR® Safe- DNA Gel Stain (Thermo Fisher Scientific).
For SYBR Green I, Cq of the NTC	No or negligible Cqs.
Evidence of optimisation (from gradients)	No standard curves were made.
Standard curves with slope and y-intercept	
PCR efficiency calculated from slope	PCR efficiency was obtained directly from the Amplification Factor values of each PCR reaction from the Comparative Quantitation application of the Rotor-Gene Q Series Software 1.7 (Build 94) Copyright ® 2008 Corbett Life Science. PCR Reaction Amplification Factors were from 1.67 to 1.96 and the Reaction Amplification Std. deviations were from 0.04 to 0.3.
Confidence interval for PCR efficiency or standard error	Not analyzed.
r2 of standard curve	No standard curves were made.
Linear dynamic range	
Cq variation at lower limit	
Confidence intervals throughout range	
Evidence for limit of detection	Cq values obtained after cycle 40 were considered out of accurate quantifiable range.
If multiplex, efficiency and LOD of each assay.	Multiplex qPCR was not performed.
DATA ANALYSIS	

qPCR analysis program (source, version)	Rotor-Gene Q Series Software 1.7 (Build 94) Copyright ® 2008 Corbett Life Science and Biogazelle qBASE+ software (Vandesompele et al. 2002).
Cq method determination	Software default settings.
Outlier identification and disposition	No data have been excluded from the calculations.
Results of NTCs	No or negligible Cqs were obtained from NTC. No prominent peaks were visualized in the melting curves.
Justification of number and choice of reference genes	Provided in the methods section.
Description of normalization method	Provided in the methods section.
Number and concordance of biological replicates	2. The Cq values were similar between the 2 biological replicates.
Number and stage (RT or qPCR) of technical replicates	2 retro transcriptions were made of each sample and 3 technical replicates were developed of each qPCR.
Repeatability (intra-assay variation)	To check reprducibility, we performed qPCR of the TUB gene using 6 technical replicates in the cDNA samples of leaves as vegetative tissue sample and embrios as reproductive tissue ones. Difference in Cqs were between 0-0.8 (0.3-1.14% CV) among the qPCR replicates. Moreover, the expression levels of the genes analyzed were confirmed to be similar between 2 biological replicates.
Reproducibility (inter-assay variation, %CV)	
Power analysis	Statistical and mathematical analysis was developed with three different software. geNorm from Biogazelle qBASE+ software (Vandesompele et al. 2002), and two publicly available software applications, BestKeeper (Pfaffl et al. 2004) and NormFinder (Andersen et al. 2004).
Statistical methods for result significance	
Software (source, version)	
Cq or raw data submission using RDML	None

References

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